

**PRODUCTION OF GAMMA LINOLENIC ACID**  
**BY A Δ6-DESATURASE**

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This is a continuation-in-part of U.S. Serial  
No. 08/789,936 filed January 28, 1997<sup>now US Patent 5,789,220</sup> which is a  
continuation-in-part of U.S. Serial No. 08/307,382,  
5 filed September 14, 1994<sup>now US Patent 5,552,306</sup> which is a continuation of U.S.  
Serial No. 07/959,952 filed October 13, 1992<sup>now abandoned</sup> which is a  
continuation-in-part of U.S. Serial No. 07/817,919, filed  
January 8, 1992<sup>now abandoned</sup> which is a continuation-in-part  
application of U.S. Serial No. 07/774,475 filed October 10,  
10 1991<sup>now abandoned</sup>

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**FIELD OF THE INVENTION**

Linoleic acid (18:2) (LA) is transformed into  
gamma linolenic acid (18:3) (GLA) by the enzyme Δ6-  
15 desaturase. When this enzyme, or the nucleic acid  
encoding it, is transferred into LA-producing cells, GLA  
is produced. The present invention provides nucleic  
acids comprising the Δ6-desaturase gene. More  
specifically, the nucleic acids comprise the promoters,  
20 coding regions and termination regions of the Δ6-  
desaturase genes. The present invention is further  
directed to recombinant constructions comprising a Δ6-  
desaturase coding region in functional combination with  
heterologous regulatory sequences. The nucleic acids  
25 and recombinant constructions of the instant invention  
are useful in the production of GLA in transgenic  
organisms.

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**BACKGROUND OF THE INVENTION**

1           Unsaturated fatty acids such as linoleic  
  ( $C_{18}\Delta^{9,12}$ ) and  $\alpha$ -linolenic ( $C_{18}\Delta^{9,12,15}$ ) acids are essential  
  dietary constituents that cannot be synthesized by  
  vertebrates since vertebrate cells can introduce double  
5 bonds at the  $\Delta^9$  position of fatty acids but cannot  
  introduce additional double bonds between the  $\Delta^9$  double  
  bond and the methyl-terminus of the fatty acid chain.  
  Because they are precursors of other products, linoleic  
  and  $\alpha$ -linolenic acids are essential fatty acids, and are  
10 usually obtained from plant sources. Linoleic acid can  
  be converted by mammals into  $\gamma$ -linolenic acid (GLA,  
   $C_{18}\Delta^{6,9,12}$ ) which can in turn be converted to arachidonic  
  acid (20:4), a critically important fatty acid since it  
  is an essential precursor of most prostaglandins.  
15           The dietary provision of linoleic acid, by  
  virtue of its resulting conversion to GLA and  
  arachidonic acid, satisfies the dietary need for GLA and  
  arachidonic acid. However, a relationship has been  
  demonstrated between consumption of saturated fats and  
20 health risks such as hypercholesterolemia,  
  atherosclerosis and other clinical disorders which  
  correlate with susceptibility to coronary disease, while  
  the consumption of unsaturated fats has been associated  
  with decreased blood cholesterol concentration and  
25 reduced risk of atherosclerosis. The therapeutic  
  benefits of dietary GLA may result from GLA being a  
  precursor to arachidonic acid and thus subsequently  
  contributing to prostaglandin synthesis. Accordingly,

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consumption of the more unsaturated GLA, rather than  
1 linoleic acid, has potential health benefits. However,  
GLA is not present in virtually any commercially grown  
crop plant.

Linoleic acid is converted into GLA by the  
5 enzyme  $\Delta 6$ -desaturase.  $\Delta 6$ -desaturase, an enzyme of more  
than 350 amino acids, has a membrane-bound domain and an  
active site for desaturation of fatty acids. When this  
enzyme is transferred into cells which endogenously  
produce linoleic acid but not GLA, GLA is produced. The  
10 present invention, by providing genes encoding  $\Delta 6$ -  
desaturase, allows the production of transgenic  
organisms which contain functional  $\Delta 6$ -desaturase and  
which produce GLA. In addition to allowing production  
of large amounts of GLA, the present invention provides  
15 new dietary sources of GLA.

#### SUMMARY OF THE INVENTION

The present invention is directed to isolated  
 $\Delta 6$ -desaturase genes. Specifically, the isolated genes  
20 comprise the  $\Delta 6$ -desaturase promoters, coding regions,  
and termination regions.

The present invention is further directed to  
expression vectors comprising the  $\Delta 6$ -desaturase  
promoter, coding region and termination region.

25 Yet another aspect of this invention is  
directed to expression vectors comprising a  $\Delta 6$ -  
desaturase coding region in functional combination with

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heterologous regulatory regions, i.e. elements not  
1 derived from the  $\Delta 6$ -desaturase gene.

Cells and organisms comprising the vectors of  
the present invention, and progeny of such organisms,  
are also provided by the present invention.

5 A further aspect of the present invention  
provides isolated bacterial  $\Delta 6$ -desaturase. Isolated  
plant  $\Delta 6$ -desaturases are also provided.

Yet another aspect of this invention provides  
a method for producing plants with increased gamma  
10 linolenic acid content.

A method for producing chilling tolerant  
plants is also provided by the present invention.

#### **BRIEF DESCRIPTION OF THE DRAWINGS:**

15 Fig. 1 depicts the hydropathy profiles of the  
deduced amino acid sequences of Synechocystis  $\Delta 6$ -  
desaturase (Panel A) and  $\Delta 12$ -desaturase (Panel B).  
Putative membrane spanning regions are indicated by  
solid bars. Hydrophobic index was calculated for a  
20 window size of 19 amino acid residues [Kyte, et al.  
(1982) J. Molec. Biol. 157].

Fig. 2 provides gas liquid chromatography  
profiles of wild type (Panel A) and transgenic (Panel B)  
Anabaena.

25 Fig. 3 is a diagram of maps of cosmid cSy75,  
cSy13 and Csy7 with overlapping regions and subclones.  
The origins of subclones of Csy75, Csy75-3.5 and Csy7

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are indicated by the dashed diagonal lines. Restriction sites that have been inactivated are in parentheses.

Fig. 4 provides gas liquid chromatography profiles of wild type (Panel A) and transgenic (Panel B) tobacco.

Fig. 5A depicts the DNA sequence of a  $\Delta 6$ -desaturase cDNA isolated from borage.

Fig. 5B depicts the protein sequence of the open reading frame in the isolated borage  $\Delta 6$ -desaturase cDNA. Three amino acid motifs characteristic of desaturases are indicated and are, in order, lipid box, metal box 1, and metal box 2.

Fig. 6 is a dendrogram showing similarity of the borage  $\Delta 6$ -desaturase to other membrane-bound desaturases. The amino acid sequence of the borage  $\Delta 6$ -desaturase was compared to other known desaturases using Gene Works (IntelliGenetics). Numerical values correlate to relative phylogenetic distances between subgroups compared.

Fig. 7 is a restriction map of 221. $\Delta 6$ .NOS and 121. $\Delta 6$ .NOS. In 221. $\Delta 6$ .NOS, the remaining portion of the plasmid is pBI221 and in 121. $\Delta 6$ .NOS, the remaining portion of the plasmid is pBI121.

Fig. 8 provides gas liquid chromatography profiles of mock transfected (Panel A) and 221. $\Delta 6$ .NOS transfected (Panel B) carrot cells. The positions of 18:2, 18:3  $\alpha$ , and 18:3  $\gamma$  (GLA) are indicated.

Fig. 9 provides gas liquid chromatography profiles of an untransformed tobacco leaf (Panel A) and

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a tobacco leaf transformed with 121.Δ6.NOS. The  
1 positions of 18:2, 18:3 a, 18:3γ (GLA), and 18:4 are  
indicated.

Fig. 10 is the complete DNA sequence and  
deduced amino acid sequence of evening primrose Δ6-  
5 desaturase. A heme binding motif of cytochrome b5  
proteins is indicated by underlined bold text.  
Underlined plain text indicates three histine rich  
motifs (HRMs). The motifs in this sequence are  
identical to those found in borage Δ6-desaturase with  
10 the exception of those that are italicized (S 161 and L  
374).

Fig. 11 is a formatted alignment of the  
evening primrose and borage Δ6-desaturase amino acid  
sequences.

15 Fig. 12A is a Kyte-Doolittle hydrophobicity  
plot for borage Δ6-desaturase.

Fig. 12B is a Kyte-Doolittle hydrophobicity  
plot for evening primrose Δ6-desaturase.

20 Fig. 13A is a Hopwood hydrophobicity plot for  
borage Δ6-desaturase. The y axis is a normalized  
parameter that estimates hydrophobicity; that the x axis  
represents the linear amino acid sequences.

Fig. 13B is a Hopwood hydrophobicity plot for  
evening primrose Δ6-desaturase. X and y axes are as in  
25 Figure 13A.

Fig. 14A graphically depicts the location of  
the transmembrane regions for borage Δ6-desaturase.  
Positive values (y-axis) greater than 500 are considered

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significant predictors of a membrane spanning region.

1 The x-axis represents the linear amino acid sequences.

Fig. 14B graphically depicts the location of the transmembrane regions for evening primrose  $\Delta 6$ -desaturase. X and y axes are as in Figure 14A.

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**DETAILED DESCRIPTION OF THE INVENTION:**

The present invention provides isolated nucleic acids encoding  $\Delta 6$ -desaturase. To identify a nucleic acid encoding  $\Delta 6$ -desaturase, DNA is isolated from an organism which produces GLA. Said organism can be, for example, an animal cell, certain fungi (e.g. Mortierella), certain bacteria (e.g. Synechocystis) or certain plants (borage, Oenothera, currants). The isolation of genomic DNA can be accomplished by a variety of methods well-known to one of ordinary skill in the art, as exemplified by Sambrook et al. (1989) in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY. The isolated DNA is fragmented by physical methods or enzymatic digestion and cloned into an appropriate vector, e.g. a bacteriophage or cosmid vector, by any of a variety of well-known methods which can be found in references such as Sambrook et al. (1989). Expression vectors containing the DNA of the present invention are specifically contemplated herein.

25 DNA encoding  $\Delta$ -desaturase can be identified by gain of function analysis. The vector containing fragmented DNA is transferred, for example by infection, transconjugation, transfection, into a host organism

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that produces linoleic acid but not GLA. As used  
1 herein, "transformation" refers generally to the  
incorporation of foreign DNA into a host cell. Methods  
for introducing recombinant DNA into a host organism are  
known to one of ordinary skill in the art and can be  
5 found, for example, in Sambrook et al. (1989).  
Production of GLA by these organisms (i.e., gain of  
function) is assayed, for example by gas chromatography  
or other methods known to the ordinarily skilled  
artisan. Organisms which are induced to produce GLA,  
10 i.e. have gained function by the introduction of the  
vector, are identified as expressing DNA encoding  $\Delta$ -  
desaturase, and said DNA is recovered from the  
organisms. The recovered DNA can again be fragmented,  
cloned with expression vectors, and functionally  
15 assessed by the above procedures to define with more  
particularity the DNA encoding  $\Delta 6$ -desaturase.

As an example of the present invention, random  
DNA is isolated from the cyanobacteria Synechocystis  
Pasteur Culture Collection (PCC) 6803, American Type  
20 Culture Collection (ATCC) 27184, cloned into a cosmid  
vector, and introduced by transconjugation into the GLA-  
deficient Cyanobacterium Anabaena strain PCC 7120, ATCC  
27893. Production of GLA from Anabaena linoleic acid is  
monitored by gas chromatography and the corresponding  
25 DNA fragment is isolated.

The isolated DNA is sequenced by methods well-  
known to one of ordinary skill in the art as found, for  
example, in Sambrook et al. (1989).

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In accordance with the present invention, DNA molecules comprising  $\Delta 6$ -desaturase genes have been isolated. More particularly, a 3.588 kilobase (kb) DNA comprising a  $\Delta 6$ -desaturase gene has been isolated from the cyanobacteria Synechocystis. The nucleotide sequence of the 3.588 kb DNA was determined and is shown in SEQ ID NO:1. Open reading frames defining potential coding regions are present from nucleotide 317 to 1507 and from nucleotide 2002 to 3081. To define the nucleotides responsible for encoding  $\Delta 6$ -desaturase, the 3.588 kb fragment that confers  $\Delta 6$ -desaturase activity is cleaved into two subfragments, each of which contains only one open reading frame. Fragment ORF1 contains nucleotides 1 through 1704, while fragment ORF2 contains nucleotides 1705 through 3588. Each fragment is subcloned in both forward and reverse orientations into a conjugal expression vector (AM542, Wolk et al. [1984] Proc. Natl. Acad. Sci. USA 81, 1561) that contains a cyanobacterial carboxylase promoter. The resulting constructs (i.e. ORF1(F), ORF1(R), ORF2(F) and ORF2(R)) are conjugated to wild-type Anabaena PCC 7120 by standard methods (see, for example, Wolk et al. (1984) Proc. Natl. Acad. Sci. USA 81, 1561). Conjugated cells of Anabaena are identified as Neo<sup>R</sup> green colonies on a brown background of dying non-conjugated cells after two weeks of growth on selective media (standard mineral media BG11N + containing 30 $\mu$ g/ml of neomycin according to Rippka et al., (1979) J. Gen Microbiol. 111, 1). The green colonies are selected and grown in selective

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liquid media (BG11N + with 15µg/ml neomycin). Lipids  
 1 are extracted by standard methods (e.g. Dahmer et al.,  
 (1989) Journal of American Oil Chemical Society 66, 543)  
 from the resulting transconjugants containing the  
 forward and reverse oriented ORF1 and ORF2 constructs.  
 5 For comparison, lipids are also extracted from wild-type  
 cultures of Anabaena and Synechocystis. The fatty acid  
 methyl esters are analyzed by gas liquid chromatography  
 (GLC), for example with a Tracor-560 gas liquid  
 chromatograph equipped with a hydrogen flame ionization  
 10 detector and a capillary column. The results of GLC  
 analysis are shown in Table 1.

Table 1: Occurrence of C18 fatty acids in wild-type and  
 transgenic cyanobacteria

SOURCE	18:0	18:1	18:2	18:3	18:4	18:5
Anabaena (wild type)	+	+	+	-	+	-
Anabaena + ORF1 (F)	+	+	+	-	+	-
Anabaena + ORF1 (R)	+	+	+	-	+	-
Anabaena + ORF2 (F)	+	+	+	+	+	+
Anabaena + ORF2 (R)	+	+	+	-	+	-
Synechocystis (wild type)	+	+	+	+	-	-

As assessed by GLC analysis, GLA deficient  
Anabaena gain the function of GLA production when the  
 construct containing ORF2 in forward orientation is  
 introduced by transconjugation. Transconjugants

containing constructs with ORF2 in reverse orientation  
1 to the carboxylase promoter, or ORF1 in either  
orientation, show no GLA production. This analysis  
demonstrates that the single open reading frame (ORF2)  
within the 1884 bp fragment encodes  $\Delta 6$ -desaturase. The  
5 1884 bp fragment is shown as SEQ ID NO:3. This is  
substantiated by the overall similarity of the  
hydropathy profiles between  $\Delta 6$ -desaturase and  $\Delta 12$ -  
desaturase [Wada et al. (1990) Nature 347] as shown in  
Fig. 1 as (A) and (B), respectively.

10 Also in accordance with the present invention,  
a cDNA comprising a  $\Delta 6$ -desaturase gene from borage  
(Borago officinalis) has been isolated. The nucleotide  
sequence of the 1.685 kilobase (kb) cDNA was determined  
and is shown in Fig. 5A (SEQ ID NO: 4). The ATG start  
15 codon and stop codon are underlined. The amino acid  
sequence corresponding to the open reading frame in the  
borage delta 6-desaturase is shown in Fig. 5B (SEQ ID  
NO: 5).

20 Additionally, the present invention provides a  
 $\Delta 6$ -desaturase gene from evening primrose (Oenothera  
biennis). The nucleotide sequence of the 1.687 kb cDNA  
was determined and is depicted in Figure 10 (SEQ ID  
NO:26). Also shown in Figure 10 is the deduced amino  
acid sequence of evening primrose  $\Delta 6$ -desaturase.

25 Isolated nucleic acids encoding  $\Delta 6$ -desaturase  
can be identified from other GLA-producing organisms by  
the gain of function analysis described above, or by  
nucleic acid hybridization techniques using the isolated

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1 nucleic acid which encodes Synechocystis, borage, or  
evening primrose  $\Delta 6$ -desaturase as a hybridization probe.  
Both methods are known to the skilled artisan and are  
contemplated by the present invention. The  
hybridization probe can comprise the entire DNA sequence  
5 disclosed as SEQ. ID NO:1 or SEQ. ID NO:4, or a  
restriction fragment or other DNA fragment thereof,  
including an oligonucleotide probe. Methods for cloning  
homologous genes by cross-hybridization are known to the  
ordinarily skilled artisan and can be found, for  
10 example, in Sambrook (1989) and Beltz et al. (1983)  
Methods in Enzymology 100, 266.

In another method of identifying a delta 6-  
desaturase gene from an organism producing GLA, a cDNA  
library is made from poly-A<sup>+</sup> RNA isolated from polysomal  
15 RNA. In order to eliminate hyper-abundant expressed  
genes from the cDNA population, cDNAs or fragments  
thereof corresponding to hyper-abundant cDNAs genes are  
used as hybridization probes to the cDNA library. Non  
hybridizing plaques are excised and the resulting  
20 bacterial colonies are used to inoculate liquid cultures  
and sequenced. For example, as a means of eliminating  
other seed storage protein cDNAs from a cDNA library  
made from borage polysomal RNA, cDNAs corresponding to  
abundantly expressed seed storage proteins are first  
25 hybridized to the cDNA library. The "subtracted" DNA  
library is then used to generate expressed sequence tags  
(ETs) and such tags are used to scan a data base such  
as GenBank to identify potential desaturates.

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Using another method, an evening primrose cDNA  
1 may be isolated by first synthesizing sequences from the  
borage  $\Delta 6$ -desaturase gene and then using these sequences  
as primers in a PCR reaction with the evening primrose  
cDNA library serving as template. PCR fragments of  
5 expected size may then be used to screen an evening  
primrose cDNA library. Hybridizing clones may then be  
sequenced and compared to the borage cDNA sequence to  
determine if the hybridizing clone represents an evening  
primrose  $\Delta 6$ -desaturase gene.

10 Transgenic organisms which gain the function  
of GLA production by introduction of DNA encoding  $\Delta 6$ -  
desaturase also gain the function of octadecatetraenoic  
acid ( $18:4^{6,9,12,15}$ ) production. Octadecatetraenoic acid  
is present normally in fish oils and in some plant  
15 species of the Boraginaceae family (Craig et al. [1964]  
J. Amer. Oil Chem. Soc. 41, 209-211; Gross et al. [1976]  
Can. J. Plant Sci. 56, 659-664). In the transgenic  
organisms of the present invention, octadecatetraenoic  
acid results from further desaturation of  $\alpha$ -linolenic  
20 acid by  $\Delta 6$ -desaturase or desaturation of GLA by  $\Delta 15$ -  
desaturase.

The 359 amino acids encoded by ORF2, i.e. the  
open reading frame encoding Synechocystis  $\Delta 6$ -desaturase,  
are shown as SEQ. ID NO:2. The open reading frame  
25 encoding the borage  $\Delta 6$ -desaturase is shown in SEQ ID NO:  
5. The present invention further contemplates other  
nucleotide sequences which encode the amino acids of SEQ  
ID NO:2 and SEQ ID NO: 5. It is within the ken of the

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ordinarily skilled artisan to identify such sequences  
1 which result, for example, from the degeneracy of the  
genetic code. Furthermore, one of ordinary skill in the  
art can determine, by the gain of function analysis  
described hereinabove, smaller subfragments of the  
5 fragments containing the open reading frames which  
encode  $\Delta 6$ -desaturases.

The present invention contemplates any such  
polypeptide fragment of  $\Delta 6$ -desaturase and the nucleic  
acids therefor which retain activity for converting LA  
10 to GLA.

In another aspect of the present invention, a  
vector containing a nucleic acid of the present  
invention or a smaller fragment containing the promoter,  
coding sequence and termination region of a  $\Delta 6$ -  
15 desaturase gene is transferred into an organism, for  
example, cyanobacteria, in which the  $\Delta 6$ -desaturase  
promoter and termination regions are functional.  
Accordingly, organisms producing recombinant  $\Delta 6$ -  
desaturase are provided by this invention. Yet another  
20 aspect of this invention provides isolated  $\Delta 6$ -  
desaturase, which can be purified from the recombinant  
organisms by standard methods of protein purification.  
(For example, see Ausubel et al. [1987] Current  
Protocols in Molecular Biology, Green Publishing  
25 Associates, New York).

Vectors containing DNA encoding  $\Delta 6$ -desaturase  
are also provided by the present invention. It will be  
apparent to one of ordinary skill in the art that

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appropriate vectors can be constructed to direct the  
1 expression of the  $\Delta 6$ -desaturase coding sequence in a  
variety of organisms. Replicable expression vectors are  
particularly preferred. Replicable expression vectors  
as described herein are DNA or RNA molecules engineered  
5 for controlled expression of a desired gene, i.e. the  
 $\Delta 6$ -desaturase gene. Preferably the vectors are  
plasmids, bacteriophages, cosmids or viruses. Shuttle  
vectors, e.g. as described by Wolk et al. (1984) Proc.  
Natl. Acad. Sci. USA, 1561-1565 and Bustos et al. (1991)  
10 J. Bacteriol. 174, 7525-7533, are also contemplated in  
accordance with the present invention. Sambrook et al.  
(1989), Goeddel, ed. (1990) Methods in Enzymology 185  
Academic Press, and Perbal (1988) A Practical Guide to  
Molecular Cloning, John Wiley and Sons, Inc., provide  
15 detailed reviews of vectors into which a nucleic acid  
encoding the present  $\Delta 6$ -desaturase can be inserted and  
expressed. Such vectors also contain nucleic acid  
sequences which can effect expression of nucleic acids  
encoding  $\Delta 6$ -desaturase. Sequence elements capable of  
20 effecting expression of a gene product include  
promoters, enhancer elements, upstream activating  
sequences, transcription termination signals and  
polyadenylation sites. The upstream 5' untranslated  
region of the evening primrose  $\Delta 6$ -desaturase gene as  
25 depicted in Figure 10 may also be used. Both  
constitutive and tissue specific promoters are  
contemplated. For transformation of plant cells, the  
cauliflower mosaic virus (CaMV) 35S promoter, other

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constitutive promoters and promoters which are regulated  
1 during plant seed maturation are of particular interest.  
All such promoter and transcriptional regulatory  
elements, singly or in combination, are contemplated for  
use in the present replicable expression vectors and are  
5 known to one of ordinary skill in the art. The CaMV 35S  
promoter is described, for example, by Restrepo et al.  
(1990) Plant Cell 2, 987. Genetically engineered and  
mutated regulatory sequences are also contemplated.

The ordinarily skilled artisan can determine  
10 vectors and regulatory elements suitable for expression  
in a particular host cell. For example, a vector  
comprising the promoter from the gene encoding the  
carboxylase of Anabaena operably linked to the coding  
region of  $\Delta 6$ -desaturase and further operably linked to a  
15 termination signal from Synechocystis is appropriate for  
expression of  $\Delta 6$ -desaturase in cyanobacteria. "Operably  
linked" in this context means that the promoter and  
terminator sequences effectively function to regulate  
transcription. As a further example, a vector  
20 appropriate for expression of  $\Delta 6$ -desaturase in  
transgenic plants can comprise a seed-specific promoter  
sequence derived from helianthinin, napin, or glycinin  
operably linked to the  $\Delta 6$ -desaturase coding region and  
further operably linked to a seed <sup>-specific</sup> termination signal or  
25 the nopaline synthase termination signal. As a still  
further example, a vector for use in expression of  $\Delta 6$ -  
desaturase in plants can comprise a constitutive  
promoter or a tissue specific promoter operably linked

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to the  $\Delta 6$ -desaturase coding region and further operably  
1 linked to a constitutive or tissue specific terminator  
or the nopaline synthase termination signal.

In particular, the helianthinin regulatory  
elements disclosed in applicant's copending U.S. <sup>A'</sup>  
Application Serial No. 682,354, filed April 8, 1991<sup>A'</sup> and  
5 incorporated herein by reference, are contemplated as  
promoter elements to direct the expression of the  $\Delta 6$ -  
desaturases of the present invention. The albumin  
regulatory elements disclosed in applicant's copending  
10 U.S. application Serial No. 08/831,570<sup>A'</sup> and the oleosin  
regulatory elements disclosed in applicant's copending  
U.S. application Serial No. 08/831,575<sup>A'</sup> (both applications  
filed April 9, 1997), and incorporated herein by  
reference, are also contemplated as elements to direct  
15 the expression of the  $\Delta 6$ -desaturases of the present  
invention.

Modifications of the nucleotide sequences or  
regulatory elements disclosed herein which maintain the  
functions contemplated herein are within the scope of  
20 this invention. Such modifications include insertions,  
substitutions and deletions, and specifically  
substitutions which reflect the degeneracy of the  
genetic code.

Standard techniques for the construction of  
25 such hybrid vectors are well-known to those of ordinary  
skill in the art and can be found in references such as  
Sambrook et al. (1989), or any of the myriad of  
laboratory manuals on recombinant DNA technology that

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are widely available. A variety of strategies are  
1 available for ligating fragments of DNA, the choice of  
which depends on the nature of the termini of the DNA  
fragments. It is further contemplated in accordance  
with the present invention to include in the hybrid  
5 vectors other nucleotide sequence elements which  
facilitate cloning, expression or processing, for  
example sequences encoding signal peptides, a sequence  
encoding KDEL or related sequence, which is required for  
retention of proteins in the endoplasmic reticulum or  
10 sequences encoding transit peptides which direct  $\Delta 6$ -  
desaturase to the chloroplast. Such sequences are known  
to one of ordinary skill in the art. An optimized  
transit peptide is described, for example, by Van den  
Broeck *et al.* (1985) Nature 313, 358. Prokaryotic and  
15 eukaryotic signal sequences are disclosed, for example,  
by Michaelis *et al.* (1982) Ann. Rev. Microbiol. 36, 425.

A further aspect of the instant invention  
provides organisms other than cyanobacteria or plants  
which contain the DNA encoding the  $\Delta 6$ -desaturase of the  
20 present invention. The transgenic organisms  
contemplated in accordance with the present invention  
include bacteria, cyanobacteria, fungi, and plants and  
animals. The isolated DNA of the present invention can  
be introduced into the host by methods known in the art,  
25 for example infection, transfection, transformation or  
transconjugation. Techniques for transferring the DNA  
of the present invention into such organisms are widely

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known and provided in references such as Sambrook et al.  
1 (1989).

A variety of plant transformation methods are  
known. The  $\Delta 6$ -desaturase gene can be introduced into  
plants by a leaf disk transformation-regeneration  
5 procedure as described by Horsch et al. (1985) Science  
227, 1229. Other methods of transformation, such as  
protoplast culture (Horsch et al. (1984) Science 223,  
496; DeBlock et al. (1984) EMBO J. 2, 2143; Barton et  
al. (1983) Cell 32, 1033) can also be used and are  
10 within the scope of this invention. In a preferred  
embodiment plants are transformed with Agrobacterium-  
derived vectors such as those described in Klett et al.  
(1987) Annu. Rev. Plant Physiol. 38:467. However, other  
15 methods are available to insert the  $\Delta 6$ -desaturase genes  
of the present invention into plant cells. Such  
alternative methods include biolistic approaches (Klein  
et al. (1987) Nature 327, 70), electroporation,  
chemically-induced DNA uptake, and use of viruses or  
pollen as vectors.

20 When necessary for the transformation method,  
the  $\Delta 6$ -desaturase genes of the present invention can be  
inserted into a plant transformation vector, e.g. the  
binary vector described by Bevan (1984) Nucleic Acids  
Res. 12, 8111. Plant transformation vectors can be  
25 derived by modifying the natural gene transfer system of  
Agrobacterium tumefaciens. The natural system comprises  
large Ti (tumor-inducing)-plasmids containing a large  
segment, known as T-DNA, which is transferred to

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transformed plants. Another segment of the Ti plasmid,  
1 the vir region, is responsible for T-DNA transfer. The  
T-DNA region is bordered by terminal repeats. In the  
modified binary vectors the tumor-inducing genes have  
been deleted and the functions of the vir region are  
5 utilized to transfer foreign DNA bordered by the T-DNA  
border sequences. The T-region also contains a  
selectable marker for antibiotic resistance, and a  
multiple cloning site for inserting sequences for  
transfer. Such engineered strains are known as  
10 "disarmed" A. tumefaciens strains, and allow the  
efficient transformation of sequences bordered by the T-  
region into the nuclear genomes of plants.

Surface-sterilized leaf disks are inoculated  
with the "disarmed" foreign DNA-containing A.  
15 tumefaciens, cultured for two days, and then transferred  
to antibiotic-containing medium. Transformed shoots are  
selected after rooting in medium containing the  
appropriate antibiotic, transferred to soil and  
regenerated.

20 Another aspect of the present invention  
provides transgenic plants or progeny of these plants  
containing the isolated DNA of the invention. Both  
monocotyledenous and dicotyledenous plants are  
contemplated. Plant cells are transformed with the  
25 isolated DNA encoding  $\Delta 6$ -desaturase by any of the plant  
transformation methods described above. The transformed  
plant cell, usually in a callus culture or leaf disk, is  
regenerated into a complete transgenic plant by methods

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well-known to one of ordinary skill in the art (e.g.  
1 Horsch et al. (1985) Science 227, 1129). In a preferred  
embodiment, the transgenic plant is sunflower, oil seed  
rape, maize, tobacco, peanut or soybean. Since progeny  
of transformed plants inherit the DNA encoding  $\Delta 6$ -  
5 desaturase, seeds or cuttings from transformed plants  
are used to maintain the transgenic plant line.

The present invention further provides a  
method for providing transgenic plants with an increased  
content of GLA. This method includes introducing DNA  
10 encoding  $\Delta 6$ -desaturase into plant cells which lack or  
have low levels of GLA but contain LA, and regenerating  
plants with increased GLA content from the transgenic  
cells. In particular, commercially grown crop plants  
are contemplated as the transgenic organism, including,  
15 but not limited to, sunflower, soybean, oil seed rape,  
maize, peanut and tobacco.

The present invention further provides a  
method for providing transgenic organisms which contain  
GLA. This method comprises introducing DNA encoding  $\Delta 6$ -  
20 desaturase into an organism which lacks or has low  
levels of GLA, but contains LA. In another embodiment,  
the method comprises introducing one or more expression  
vectors which comprise DNA encoding  $\Delta 12$ -desaturase and  
 $\Delta 6$ -desaturase into organisms which are deficient in both  
25 GLA and LA. Accordingly, organisms deficient in both LA  
and GLA are induced to produce LA by the expression of  
 $\Delta 12$ -desaturase, and GLA is then generated due to the  
expression of  $\Delta 6$ -desaturase. Expression vectors

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comprising DNA encoding  $\Delta 12$ -desaturase, or  $\Delta 12$ -  
1 desaturase and  $\Delta 6$ -desaturase, can be constructed by  
methods of recombinant technology known to one of  
ordinary skill in the art (Sambrook et al., 1989) and  
the published sequence of  $\Delta 12$ -desaturase (Wada et al  
5 [1990] Nature (London) 347, 200-203. In addition, it  
has been discovered in accordance with the present  
invention that nucleotides 2002-3081 of SEQ. ID NO:1  
encode cyanobacterial  $\Delta 12$ -desaturase. Accordingly, this  
sequence can be used to construct the subject expression  
10 vectors. In particular, commercially grown crop plants  
are contemplated as the transgenic organism, including,  
but not limited to, sunflower, soybean, oil seed rape,  
maize, peanut and tobacco.

The present invention is further directed to a  
15 method of inducing chilling tolerance in plants.  
Chilling sensitivity may be due to phase transition of  
lipids in cell membranes. Phase transition temperature  
depends upon the degree of unsaturation of fatty acids  
in membrane lipids, and thus increasing the degree of  
20 unsaturation, for example by introducing  $\Delta 6$ -desaturase  
to convert LA to GLA, can induce or improve chilling  
resistance. Accordingly, the present method comprises  
introducing DNA encoding  $\Delta 6$ -desaturase into a plant  
cell, and regenerating a plant with improved chilling  
25 resistance from said transformed plant cell. In a  
preferred embodiment, the plant is a sunflower, soybean,  
oil seed rape, maize, peanut or tobacco plant.

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The following examples further illustrate the  
1 present invention.

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**EXAMPLE 1**

**1 Strains and Culture Conditions**

Synechocystis (PCC 6803, ATCC 27184), Anabaena (PCC 7120, ATCC 27893) and Synechococcus (PCC 7942, ATCC 33912) were grown photoautotrophically at 30°C in BG11N+ medium (Rippka et al. [1979] J. Gen. Microbiol. 111, 1-61) under illumination of incandescent lamps ( $60\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). Cosmids and plasmids were selected and propagated in Escherichia coli strain DH5 $\alpha$  on LB medium supplemented with antibiotics at standard concentrations as described by Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring, New York.

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**EXAMPLE 2**

**1 Construction of Synechocystis Cosmid Genomic Library**

Total genomic DNA from Synechocystis (PCC 6803) was partially digested with Sau3A and fractionated  
5 on a sucrose gradient (Ausubel et al. [1987] Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York). Fractions containing 30 to 40 kb DNA fragments were selected and ligated into the dephosphorylated BamHI site of the  
10 cosmid vector, pDUCA7 (Buikema et al. [1991] J. Bacteriol. 173, 1879-1885). The ligated DNA was packaged in vitro as described by Ausubel et al. (1987), and packaged phage were propagated in E. coli DH5 $\alpha$  containing the AvaI and Eco4711 methylase helper  
15 plasmid, pRL528 as described by Buikema et al. (1991). A total of 1152 colonies were isolated randomly and maintained individually in twelve 96-well microtiter plates.

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**EXAMPLE 3**

**1 Gain-of-Function Expression of GLA in Anabaena**

Anabaena (PCC 7120), a filamentous cyanobacterium, is deficient in GLA but contains  
5 significant amounts of linoleic acid, the precursor for GLA (Figure 2; Table 2). The Synechocystis cosmid library described in Example 2 was conjugated into Anabaena (PCC 7120) to identify transconjugants that  
10 produce GLA. Anabaena cells were grown to mid-log phase in BG11N+ liquid medium and resuspended in the same medium to a final concentration of approximately  $2 \times 10^8$  cells per ml. A mid-log phase culture of E. coli RP4 (Burkardt et al. [1979] J. Gen. Microbiol. 114, 341-348) grown in LB containing ampicillin was washed and  
15 resuspended in fresh LB medium. Anabaena and RP4 were then mixed and spread evenly on BG11N+ plates containing 5% LB. The cosmid genomic library was replica plated onto LB plates containing 50 µg/ml kanamycin and 17.5 µg/ml chloramphenicol and was subsequently patched onto  
20 BG11N+ plates containing Anabaena and RP4. After 24 hours of incubation at 30°C, 30 µg/ml of neomycin was underlaid; and incubation at 30°C was continued until transconjugants appeared.

Individual transconjugants were isolated after  
25 conjugation and grown in 2 ml BG11N+ liquid medium with 15 µg/ml neomycin. Fatty acid methyl esters were prepared from wild type cultures and cultures containing pools of ten transconjugants as follows. Wild type and

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transgenic cyanobacterial cultures were harvested by  
1 centrifugation and washed twice with distilled water.  
Fatty acid methyl esters were extracted from these  
cultures as described by Dahmer et al. (1989) J. Amer.  
Oil. Chem. Soc. 66, 543-548 and were analyzed by Gas  
5 Liquid Chromatography (GLC) using a Tracor-560 equipped  
with a hydrogen flame ionization detector and capillary  
column (30 m x 0.25 mm bonded FSOT Superox II, Alltech  
Associates Inc., IL). Retention times and co-  
chromatography of standards (obtained from Sigma  
10 Chemical Co.) were used for identification of fatty  
acids. The average fatty acid composition was  
determined as the ratio of peak area of each C18 fatty  
acid normalized to an internal standard.

Representative GLC profiles are shown in Fig.  
15 2. C18 fatty acid methyl esters are shown. Peaks were  
identified by comparing the elution times with known  
standards of fatty acid methyl esters and were confirmed  
by gas chromatography-mass spectrometry. Panel A  
depicts GLC analysis of fatty acids of wild type  
20 Anabaena. The arrow indicates the migration time of  
GLA. Panel B is a GLC profile of fatty acids of  
transconjugants of Anabaena with pAM542+1.8F. Two GLA  
producing pools (of 25 pools representing 250  
transconjugants) were identified that produced GLA.  
25 Individual transconjugants of each GLA positive pool  
were analyzed for GLA production; two independent  
transconjugants, AS13 and AS75, one from each pool, were  
identified which expressed significant levels of GLA and

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which contained cosmids, cSy13 and cSy75, respectively  
1 (Figure 3). The cosmids overlap in a region  
approximately 7.5 kb in length. A 3.5 kb NheI fragment  
of cSy75 was recloned in the vector pDUCA7 and  
transferred to Anabaena resulting in gain-of-function  
5 expression of GLA (Table 2).

Two NheI/Hind III subfragments (1.8 and 1.7  
kb) of the 3.5 kb Nhe I fragment of cSy75-3.5 were  
subcloned into "pBLUESCRIPT" (Stratagene) (Figure 3) for  
sequencing. Standard molecular biology techniques were  
10 performed as described by Maniatis et al. (1982) and  
Ausubel et al. (1987). Dideoxy sequencing (Sanger et al.  
[1977] Proc. Natl. Acad. Sci. USA 74, 5463-5467) of  
pBS1.8 was performed with "SEQUENASE" (United States  
Biochemical) on both strands by using specific  
15 oligonucleotide primers synthesized by the Advanced DNA  
Technologies Laboratory (Biology Department, Texas A & M  
University). DNA sequence analysis was done with the  
GCG (Madison, WI) software as described by Devereux et  
al. (1984) Nucleic Acids Res. 12, 387-395.

20 Both NheI/HindIII subfragments were  
transferred into a conjugal expression vector, AM542, in  
both forward and reverse orientations with respect to a  
cyanobacterial carboxylase promoter and were introduced  
into Anabaena by conjugation. Transconjugants  
25 containing the 1.8 kb fragment in the forward  
orientation (AM542-1.8F) produced significant quantities  
of GLA and octadecatetraenoic acid (Figure 2; Table 2).  
Transconjugants containing other constructs, either

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reverse oriented 1.8 kb fragment or forward and reverse  
1 oriented 1.7 kb fragment, did not produce detectable  
levels of GLA (Table 2).

Figure 2 compares the C18 fatty acid profile  
of an extract from wild type Anabaena (Figure 2A) with  
5 that of transgenic Anabaena containing the 1.8 kb  
fragment of cSy75-3.5 in the forward orientation (Figure  
2B). GLC analysis of fatty acid methyl esters from  
AM542-1.8F revealed a peak with a retention time  
identical to that of authentic GLA standard. Analysis  
10 of this peak by gas chromatography-mass spectrometry  
(GC-MS) confirmed that it had the same mass  
fragmentation pattern as a GLA reference sample.  
Transgenic Anabaena with altered levels of  
polyunsaturated fatty acids were similar to wild type in  
15 growth rate and morphology.

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EXAMPLE 4

1 Transformation of Synechococcus  
with  $\Delta 6$  and  $\Delta 12$  Desaturase Genes

5 A third cosmid, cSy7, which contains a  $\Delta 12$ -  
desaturase gene, was isolated by screening the  
Synechocystis genomic library with a oligonucleotide  
synthesized from the published Synechocystis  $\Delta 12$ -  
desaturase gene sequence (Wada et al. [1990] Nature  
10 (London) 347, 200-203). A 1.7 kb AvaI fragment from  
this cosmid containing the  $\Delta 12$ -desaturase gene was  
identified and used as a probe to demonstrate that cSy13  
not only contains a  $\Delta 6$ -desaturase gene but also a  $\Delta 12$ -  
desaturase gene (Figure 3). Genomic Southern blot  
analysis further showed that both the  $\Delta 6$ -and  $\Delta 12$ -  
15 desaturase genes are unique in the Synechocystis genome  
so that both functional genes involved in C18 fatty acid  
desaturation are linked closely in the Synechocystis  
genome.

20 The unicellular cyanobacterium Synechococcus  
(PCC 7942) is deficient in both linoleic acid and  
GLA(3). The  $\Delta 12$  and  $\Delta 6$ -desaturase genes were cloned  
individually and together into pAM854 (Bustos et al.  
[1991] J. Bacteriol. 174, 7525-7533), a shuttle vector  
that contains sequences necessary for the integration of  
foreign DNA into the genome of Synechococcus (Golden et  
25 al. [1987] Methods in Enzymol. 153, 215-231).  
Synechococcus was transformed with these gene constructs  
and colonies were selected. Fatty acid methyl esters

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were extracted from transgenic Synechococcus and  
1 analyzed by GLC.

Table 2 shows that the principal fatty acids  
of wild type Synechococcus are stearic acid (18:0) and  
oleic acid (18:1). Synechococcus transformed with  
5 pAM854- $\Delta$ 12 expressed linoleic acid (18:2) in addition to  
the principal fatty acids. Transformants with pAM854- $\Delta$ 6  
and  $\Delta$ 12 produced both linoleate and GLA (Table 1).  
These results indicated that Synechococcus containing  
both  $\Delta$ 12- and  $\Delta$ 6-desaturase genes had gained the  
10 capability of introducing a second double bond at the  
 $\Delta$ 12 position and a third double bond at the  $\Delta$ 6 position  
of C18 fatty acids. However, no changes in fatty acid  
composition was observed in the transformant containing  
pAM854- $\Delta$ 6, indicating that in the absence of substrate  
15 synthesized by the  $\Delta$ 12 desaturase, the  $\Delta$ 6-desaturase is  
inactive. This experiment further confirms that the 1.8  
kb NheI/HindIII fragment (Figure 3) contains both coding  
and promoter regions of the Synechocystis  $\Delta$ 6-desaturase  
gene. Transgenic Synechococcus with altered levels of  
20 polyunsaturated fatty acids were similar to wild type in  
growth rate and morphology.

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**TABLE 2**

**Composition of C18 Fatty Acids in Wild Type and Transgenic Cyanobacteria**

Strain	Fatty acid (%)					
	18:0	18:1	18:2	18:3(α)	18:3(γ)	18:4
<b>Wild Type</b>						
<i>Synechocystis</i> (sp. PCC6803)	13.6	4.5	54.5	—	27.3	—
<i>Anabaena</i> (sp. PCC7120)	2.9	24.8	37.1	35.2	—	—
<i>Synechococcus</i> (sp. PCC7942)	20.6	79.4	—	—	—	—
<b>Anabaena Transcon-</b>						
<b>stants</b>						
<i>cy75</i>	3.8	24.4	22.3	9.1	27.9	12.5
<i>cy75-3.5</i>	4.3	27.6	18.1	3.2	40.4	6.4
pAM542 - 1.8F	4.2	13.9	12.1	19.1	25.4	25.4
pAM542 - 1.8R	7.7	23.1	38.4	30.8	—	—
pAM542 - 1.7F	2.8	27.8	36.1	33.3	—	—
pAM542 - 1.7R	2.8	25.4	42.3	29.6	—	—
<b>Synechococcus Trans-</b>						
<b>stants</b>						
pAM854	27.8	72.2	—	—	—	—
pAM854 - Δ <sup>12</sup>	4.0	43.2	46.0	—	—	—
pAM854 - Δ <sup>6</sup>	18.2	81.8	—	—	—	—
pAM854 - Δ <sup>6</sup> & Δ <sup>12</sup>	42.7	25.3	19.5	—	16.5	—

18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3(α), α-linolenic acid; 18:3(γ), γ-linolenic acid; 18:4, octadecatetraenoic acid

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**EXAMPLE 5**

**1 Nucleotide Sequence of  $\Delta 6$ -Desaturase**

5 The nucleotide sequence of the 1.8 kb fragment  
of cSy75-3.5 including the functional  $\Delta 6$ -desaturase gene  
was determined. An open reading frame encoding a  
polypeptide of 359 amino acids was identified (Figure  
4). A Kyte-Doolittle hydropathy analysis (Kyte et al.  
[1982] J. Mol. Biol. 157, 105-132) identified two  
regions of hydrophobic amino acids that could represent  
10 transmembrane domains (Figure 1A); furthermore, the  
hydropathic profile of the  $\Delta 6$ -desaturase is similar to  
that of the  $\Delta 12$ -desaturase gene (Figure 1B; Wada et al.)  
and  $\Delta 9$ -desaturases (Thiede et al. [1986] J. Biol. Chem.  
261, 13230-13235). However, the sequence similarity  
15 between the Synechocystis  $\Delta 6$ - and  $\Delta 12$ -desaturases is  
less than 40% at the nucleotide level and approximately  
18% at the amino acid level.

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**EXAMPLE 6**

**1      Transfer of Cyanobacterial  $\Delta^6$ -Desaturase into Tobacco**

5                    The cyanobacterial  $\Delta^6$ -desaturase gene was mobilized into a plant expression vector and transferred to tobacco using Agrobacterium mediated gene transfer techniques. To ensure that the transferred desaturase is appropriately expressed in leaves and developing seeds and that the desaturase gene product is targeted to the endoplasmic reticulum or the chloroplast, various expression cassettes with Synechocystis  $\Delta$ -desaturase open reading frame (ORF) were constructed. Components of these cassettes include: (i) a 35S promoter or seed specific promoter derived from the sunflower helianthinin gene to drive  $\Delta 6$ -desaturase gene expression in all plant tissues or only in developing seeds respectively, (ii) a putative signal peptide either from carrot extension gene or sunflower helianthinin gene to target newly synthesized  $\Delta 6$ -desaturase into the ER, (iii) an ER lumen retention signal sequence (KDEL) at the COOH-terminal of the  $\Delta 6$ -desaturase ORF, and (iv) an optimized transit peptide to target  $\Delta 6$  desaturase into the chloroplast. The 35S promoter is a derivative of pRTL2 described by Restrepo et al. (1990). The optimized transit peptide sequence is described by Van de Broeck et al. (1985). The carrot extensin signal peptide is described by Chen et al (1985) EMBO J. 2, 2145.

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Transgenic tobacco plants were produced  
1 containing a chimeric cyanobacterial desaturase gene,  
comprised of the Synechocystis  $\Delta 6$ -desaturase gene fused  
to an endoplasmic reticulum retention sequence (KDEL)  
and extensin signal peptide driven by the CaMV 35S  
5 promoter. PCR amplifications of transgenic tobacco  
genomic DNA indicate that the  $\Delta 6$ -desaturase gene was  
incorporated into the tobacco genome. Fatty acid methyl  
esters of leaves of these transgenic tobacco plants were  
extracted and analyzed by Gas Liquid Chromatography  
10 (GLC). These transgenic tobacco accumulated significant  
amounts of GLA (Figure 4). Figure 4 shows fatty acid  
methyl esters as determined by GLC. Peaks were  
identified by comparing the elution times with known  
standards of fatty acid methyl ester. Accordingly,  
15 cyanobacterial genes involved in fatty acid metabolism  
can be used to generate transgenic plants with altered  
fatty acid compositions.

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**EXAMPLE 7**

**Construction of Borage cDNA library**

1           Membrane bound polysomes were isolated from  
borage seeds 12 days post pollination (12 DPP) using the  
protocol established for peas by Larkins and Davies  
5 (1975 Plant Phys. 55:749-756). RNA was extracted from  
the polysomes as described by Mechler (1987 Methods in  
Enzymology 152:241-248, Academic Press).

          Poly-A+ RNA was isolated from the membrane  
bound polysomal RNA by use of Oligotex-dT beads  
10 (Qiagen). Corresponding cDNA was made using  
Stratagene's ZAP cDNA synthesis kit. The cDNA library  
was constructed in the lambda ZAP II vector (Stratagene)  
using the lambda ZAP II vector kit. The primary library  
was packaged in Gigapack II Gold packaging extract  
15 (Stratagene). The library was used to generate  
expressed sequence tags (ESTs), and sequences  
corresponding to the tags were used to scan the GenBank  
database.

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**EXAMPLE 8**  
**Hybridization Protocol**

1 Hybridization probes for screening the borage  
cDNA library were generated by using random primed DNA  
synthesis as described by Ausubel et al (1994 Current  
5 Protocols in Molecular Biology, Wiley Interscience,  
N.Y.) and corresponded to previously identified  
abundantly expressed seed storage protein cDNAs.  
Unincorporated nucleotides were removed by use of a G-50  
spin column (Boehringer Mannheim). Probe was denatured  
10 for hybridization by boiling in a water bath for 5  
minutes, then quickly cooled on ice. Filters for  
hybridization were prehybridized at 60°C for 2-4 hours  
in prehybridization solution (6XSSC [Maniatis et al 1984  
Molecular Cloning A Laboratory Manual, Cold Spring  
15 Harbor Laboratory], 1X Denharts Solution, 0.05% sodium  
pyrophosphate, 100 µg/ml denatured salmon sperm DNA).  
Denatured probe was added to the hybridization solution  
(6X SSC, 1X Denharts solution, 0.05% sodium  
pyrophosphate, 100 µg/ml denatured salmon sperm DNA) and  
20 incubated at 60°C with agitation overnight. Filters  
were washed in 4x, 2x, and 1x SET washes for 15 minutes  
each at 60°C. A 20X SET stock solution is 3M NaCl, 0.4  
M Tris base, 20 mM Na<sub>2</sub>EDTA-2H<sub>2</sub>O. The 4X SET wash was 4X  
SET, 12.5 mM PO<sub>4</sub>, pH 6.8 and 0.2% SDS. The 2X SET wash  
25 was 2X SET, 12.5 mM PO<sub>4</sub>, pH 6.8 and 0.2% SDS. The 1X SET  
wash was 1X SET, 12.5 mM PO<sub>4</sub>, pH 6.8 and 0.2% SDS.  
Filters were allowed to air dry and were then exposed to  
X-ray film for 24 hours with intensifying screens at -  
80°C.

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**EXAMPLE 9**

**Random sequencing of cDNAs from a borage seed  
(12 DPP) membrane-bound polysomal library**

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The borage cDNA library was plated at low density (500 pfu on 150 mm petri dishes). Highly prevalent seed storage protein cDNAs were "subtracted" by screening with the previously identified corresponding cDNAs. Non-hybridizing plaques were excised using Stratagene's excision protocol and reagents. Resulting bacterial colonies were used to inoculate liquid cultures and were either sequenced manually or by an ABI automated sequencer. Each cDNA was sequenced once and a sequence tag generated from 200-300 base pairs. All sequencing was performed by cycle sequencing (Epicentre). Over 300 ESTs were generated. Each sequence tag was compared to GenBank database by BLASTX computer program and a number of lipid metabolism genes, including the  $\Delta 6$ -desaturase were identified.

Database searches with a cDNA clone designated mbp-65 using BLASTX with the GenBank database resulted in a significant match to the Synechocystis  $\Delta 6$ -desaturase. It was determined however, that this clone was not a full length cDNA. A full length cDNA was isolated using mbp-65 to screen the borage membrane-bound polysomal library. The sequence of the isolated cDNA was determined (Fig. 5A, SEQ ID NO:4) and the protein sequence of the open reading frame (Fig. 5B, SEQ ID NO:5) was compared to other known desaturases using Geneworks (IntelligGenetics) protein alignment program

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Although similar to other known plant desaturases, the borage delta 6-desaturase is distinct as indicated in the dendrogram shown in Fig. 6. Furthermore, comparison of the amino acid sequences

desaturases (Table 3).  
The borage delta 6-desaturase is distinguished  
from the cyanobacterial form not only in overall  
sequence (Fig. 6) but also in the lipid box, metal box 1  
and metal box 2 amino acid motifs (Table 3). As Table 3  
indicates, all three motifs are novel in sequence. Only  
the borage delta 6-desaturase metal box 2 showed some  
relationship to the Synechocystis delta-6 desaturase  
metal box 2.

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Table 3. Comparison of common amino acid motifs in membrane-bound desaturases

Desaturase	Lipid Box	Amino Acid Motif				Metal Box 1	Metal Box 2
Borage $\Delta^6$	WIGHDAGH (SEQ. ID. NO: 6)	HNAHH (SEQ. ID. NO: 12)	FQIEHH (SEQ. ID. NO: 20)				
Synechocystis $\Delta^6$	NVGHDANH (SEQ. ID. NO: 7)	HNYLHH (SEQ. ID. NO: 13)	HQVTHH (SEQ. ID. NO: 21)				
Arab. chloroplast $\Delta^{15}$	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)				
Rice $\Delta^{15}$	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)				
Glycine chloroplast $\Delta^{15}$	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)				
Arab. fad3 ( $\Delta^{15}$ )	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)				
Brassica fad3 ( $\Delta^{15}$ )	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)				
Borage $\Delta^{12}$ (Pl-81)*	VIAHECGH (SEQ. ID. NO: 9)	HRRHH (SEQ. ID. NO: 15)	HVAHH (SEQ. ID. NO: 23)				
Arab. fad2 ( $\Delta^{12}$ )	VIAHECGH (SEQ. ID. NO: 9)	HRRHH (SEQ. ID. NO: 15)	HVAHH (SEQ. ID. NO: 23)				
Arab. chloroplast $\Delta^{12}$	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)				
Glycine plastid $\Delta^{12}$	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)				
Spinach plastidial n-6	VIGHDCAH (SEQ. ID. NO: 10)	HDQHH (SEQ. ID. NO: 17)	HIPHH (SEQ. ID. NO: 24)				
Synechocystis $\Delta^{12}$	VVGHDGCH (SEQ. ID. NO: 11)	HDHHH (SEQ. ID. NO: 18)	HIPHH (SEQ. ID. NO: 24)				
Anabaena $\Delta^{12}$	VLGHDCGH (SEQ. ID. NO: 8)	HNHHH (SEQ. ID. NO: 19)	HVPHH (SEQ. ID. NO: 25)				

\*Pl-81 is a full length cDNA which was identified by EST analysis and shows high similarity to the Arbidopsis  $\Delta^{12}$  desaturase (fad2)

\*Pl-81 is a full length cDNA which was identified by EST analysis and shows high similarity to the Arabidopsis  $\Delta^{12}$  desaturase (fad2)

**EXAMPLE 10**  
**Construction of 222.1 $\Delta^6$ NOS for transient**  
**and expression**

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The vector pBI221 (Jefferson et al. 1987  
EMBO J. 6:3901-3907) was prepared for ligation by  
digestion with BamHI and EcoICR I (Promega) which  
excises the GUS coding region leaving the 35S promoter  
and NOS terminator intact. The borage  $\Delta^6$ -desaturase  
cDNA was excised from the Bluescript plasmid  
(Stratagene) by digestion with BamHI and XhoI. The  
XhoI end was made blunt by use of the Klenow fragment.  
This fragment was then cloned into the BamHI/EcoICR I  
sites of pBI221, yielding 221.1 $\Delta^6$ NOS (Fig. 7). In  
221.1 $\Delta^6$ .NOS, the remaining portion (backbone) of the  
restriction map depicted in Fig. 7 is pBI221.

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084454-09197  
45242580

**EXAMPLE 11**

**Construction of 121.1 $\Delta^6$ .NOS for stable transformation**

1           The vector pBI121 (Jefferson et al. 1987  
EMBO J. 6:3901-3907) was prepared for ligation by  
digestion with BamHI and EcoICR I (Promega) which  
5       excises the GUS coding region leaving the 35S promoter  
and NOS terminator intact. The borage  $\Delta^6$ -desaturase  
cDNA was excised from the Bluescript plasmid  
(Stratagene) by digestion with BamHI and XhoI. The  
XhoI end was made blunt by use of the Klenow fragment.  
10       This fragment was then cloned into the BamHI/EcoICR I  
sites of pBI121, yielding 121.1 $\Delta^6$ NOS (Fig. 7). In  
121. $\Delta^6$ .NOS, the remaining portion (backbone) of the  
restriction map depicted in Fig. 7 is pBI121.

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**EXAMPLE 12**  
**Transient Expression**

1 All work involving protoplasts was performed  
in a sterile hood. One ml of packed carrot suspension  
cells were digested in 30 mls plasmolyzing solution  
5 (25 g/l KCl, 3.5 g/l CaCl<sub>2</sub>·H<sub>2</sub>O, 10mM MES, pH 5.6 and  
0.2 M mannitol) with 1% cellulase, 0.1% pectolyase,  
and 0.1% dreisalase overnight, in the dark, at room  
temperature. Released protoplasts were filtered  
10 through a 150 µm mesh and pelleted by centrifugation  
(100x g, 5 min.) then washed twice in plasmolyzing  
solution. Protoplasts were counted using a double  
chambered hemocytometer. DNA was transfected into the  
protoplasts by PEG treatment as described by Nunberg  
and Thomas (1993 Methods in Plant Molecular Biology  
15 and Biotechnology, B.R. Glick and J.E. Thompson, eds.  
pp. 241-248) using 10<sup>6</sup> protoplasts and 50-70 ug of  
plasmid DNA (221.Δ6.NOS). Protoplasts were cultured  
in 5 mls of MS media supplemented with 0.2M mannitol  
and 3 µm 2,4-D for 48 hours in the dark with shaking.

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**EXAMPLE 13**

**Stable transformation of tobacco**

1                    121.1Δ<sup>6</sup>NOS plasmid construction was used to  
transform tobacco (*Nicotiana tabacum* cv. xanthi) via  
5    Agrobacterium according to standard procedures (Horsh  
et al., 1985 Science 227: 1229-1231; Bogue et al.,  
1990 Mol. Gen. Genet. 221:49-57), except that initial  
transformants were selected on 100 ug/ml kanamycin.

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**EXAMPLE 14**

**Preparation and analysis of  
fatty acid methyl esters (FAMES)**

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Tissue from transfected protoplasts and transformed tobacco plants was frozen in liquid nitrogen and lyophilized overnight. FAMES were prepared as described by Dahmer et al (1989 J. Amer. Oil Chem. Soc. 66:543-548). In some cases, the solvent was evaporated again, and the FAMES were resuspended in ethyl acetate and extracted once with deionized water to remove any water soluble contaminants. The FAMES were analyzed by gas chromatography (GC) on a J&W Scientific DB-wax column (30 m length, 0.25 mm ID, 0.25  $\mu$ m film).

An example of a transient assay is shown in Fig. 8 which represents three independent transfections pooled together. The addition of the borage  $\Delta 6$ -desaturase cDNA corresponds with the appearance of gamma linolenic acid (GLA) which is one of the possible products of  $\Delta 6$ -desaturase. Furthermore, transgenic tobacco containing the borage  $\Delta 6$ -desaturase driven by the cauliflower mosaic virus 35S promoter also produce GLA as well as octadecaenoic acid (18:4) which is formed by the further desaturation of GLA (Fig. 9). These results indicate that the borage delta 6-desaturase gene can be used to transform plant cells to achieve altered fatty acid compositions.

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256760-1524280

# EXAMPLE 15

## Isolation of an Evening Primrose $\Delta 6$ -desaturase gene

1 Total RNA was isolated from evening primrose  
embryos using the method of Chang, Puryear, and  
Cairney (1993) *Plant Mol Biol Reporter* 11:113-116.  
5 Poly A<sup>+</sup> RNA was selected on oligotex beads (Qiagen) and  
used as a template for cDNA synthesis. The cDNA  
library was constructed in the lambda ZAP II vector  
(Stratagene) using the lambda ZAP II vector kit. The  
primary library was packaged with Gigapack II Gold  
10 packaging extract (Stratagene).

PCR primers based on sequences in the borage  
 $\Delta 6$ -desaturase gene were synthesized by a commercial  
source using standard protocols and included the  
following oligonucleotides:

15 5' AAACCAATCCATCCAAGRA 3' SEQ ID NO:27  
5' KTGGTGGAAATGGAMSCATAA 3' SEQ ID NO:28  
(R=A and G, K=G and T, M=A and C, S=G and C)

A primer that matches a region that flanks  
the insertion site of the lambda ZAP II vector was  
20 also synthesized using an ABI394 DNA synthesizer and  
standard protocols. This primer had the following  
sequence:

5' TCTAGAACTAGTGGATC 3' SEQ ID NO:29

25 An aliquot of the cDNA library was used  
directly as template in a PCR reaction using SEQ ID  
NO: 27 and SEQ ID NO:29 as primers. The reactions  
were carried out in a volume of 50  $\mu$ l using an  
annealing temperature of 50°C for 2 minutes, an

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extension temperature of 72°C for 1.5 minutes, and a melting temperature of 94°C for 1 minute for 29 cycles. A final cycle with a 2 minute annealing at 50°C and a 5 minute extension at 72°C completed the reaction. One µl from this reaction was used as a template in a second reaction using the same conditions except that the primers were SEQ ID NO:27 and SEQ ID NO:28. A DNA fragment of predicted size based on the location of the primer sequences in the the borage Δ6-desaturase cDNA was isolated.

This PCR fragment was cloned into pT7 Blue (Novagen) and used to screen the evening primrose cDNA library at low stringency conditions: The hybridization buffer used was 1% bovine serum albumin (crystalline fraction V), 1mM EDTA, 0.5 M NaHPO<sub>4</sub> pH7.2, and 7% SDS. The hybridizations were at 65°C. The wash buffer was 1mM Na<sub>2</sub>EDTA, 40 mM NaHPO<sub>4</sub> pH7.2 and 1% SDS. Primary screens were washed at 25°C. Secondary and tertiary screens were washed at 25°C, 37°C, and 42°C. One of the positively hybridizing clones that was identified in the evening primrose cDNA library was excised as a phagemid in pBluescript. The DNA sequence of the 1687 bp insert of this phagemid (pIB9748-4) was determined (Fig. 10, SEQ ID NO: 26) using the ABIPRISM™ dye terminator cycle sequencing core kit from Perkin Elmer according to the manufacturer's protocol. The sequence encodes a full length protein of 450 amino acids (SEQ ID NO:27) with a molecular weight of 51492 daltons.

Alignment of the deduced amino acid sequence with that of borage Δ6-desaturase was performed using

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the Geneworks program (Fig. 11). The evening primrose  
Δ6-desaturase protein is identical at 58% of the  
1 residues and similar at an additional 20% of the  
residues. Only two small gaps, near the carboxy  
terminal end of the protein were introduced by the  
program to obtain the alignment (Fig. 11). The two  
5 proteins were compared using two different algorithms  
that measure the hydrophobicity of regions to the  
protein. Figures 12A and 12B are Kyte-Doolittle  
hydrophobicity plots of borage Δ6-desaturase and  
evening primrose Δ6-desaturase, respectively.  
10 Figures 13A and 13B are Hopwood hydrophobicity plots  
generated in the program DNA Strider for the same  
proteins. A discussion of the algorithm used to  
generate these plots can be found in Hopp, T.P. and  
Woods, K.R. 1983 Molecular Immunology 20:483-89.  
15 Substantial similarity exists between the borage and  
evening primrose proteins using either algorithm.  
TMPredict, a program that predicts the location of  
transmembrane regions of proteins was run on the two  
sequences and again similar results were obtained  
20 (Figures 14 and 15). Several weights matrices are used  
in scoring the predictions as reported in Hofmann, K.  
and Stoffel, W. 1993 *Biol. C. Hoppe-Scyler* 347:156.  
Positive values (x-axis) greater than 500 are  
considered significant predictors of a membrane  
spanning region; the x-axis represents the linear  
25 amino acid sequences.

The membrane bound desaturases of plants  
possess three histidine rich motifs (HRMs). These  
motifs are identified in the evening primrose

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sequence and are indicated in Figure 10 by underlined  
plain text. The motifs in this sequence were  
1 identical to those found in borage  $\Delta 6$ -desaturase with  
the exception of those that are italicized (S 161 and  
L374). The borage  $\Delta 6$ -desaturase is unique among known  
membrane bound desaturases in having a cytochrome *b5*  
5 domain at the carboxy terminal end. The evening  
primrose protein encoded by pIB9748-4 also has this  
domain. The heme binding motif of cytochrome *b5*  
proteins is indicated in Figure 10 by underlined bold  
text.

10           These data indicate that a  $\Delta 6$ -desaturase  
cDNA from evening primrose has been isolated and  
characterized.

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**EXAMPLE 16**

**Construction of expression vectors for transient and stable expression of an evening primrose  $\Delta 6$ -desaturase**

The evening primrose  $\Delta 6$ -desaturase cDNA is excised from the Bluescript phagemid by digestion with Xba I and Xho I. The entire cDNA sequence including the 5' transcribed but untranslated region depicted in Figure 10 (SEQ ID NO:26) is operably linked to any one of various promoters and/or other regulatory elements in an expression vector, in order to effect transcription and translation of the  $\Delta 6$ -desaturase gene. Alternatively, the cDNA sequence depicted in Figure 10 may be trimmed at the 5' end so that the 5' transcribed but untranslated sequence is removed. The A of the ATG translational start codon is then made the first nucleotide following the promoter and/or other regulatory sequence in an expression vector.

In order to express the subject evening primrose cDNA in pBI221 (Jefferson et al. 1987 EMBO J. 6:3901-3907) the following manipulations are performed:

The plasmid pBI221 is digested with EcoICR I (Promega) or Ecl 136 II (NEB) and Xba I which excises the GUS coding region and leaves the 35S promoter and NOS terminator intact. The evening primrose  $\Delta 6$ -desaturase cDNA is excised from pIB9748-4 by digestion with Xba I and Xho I. The Xho I end is made blunt by use of the Klenow fragment. The excised gene is then cloned into the cloned into the Xba I/Eco ICR I sites of pBI221. The resulting construct is then

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transfected into carrot protoplasts. One ml of packed carrot suspension cells are digested in 30 ml of plasmolyzing solution (25 g/l KCl 3.5 g/l  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ , 10 mM MES, pH 5.6 and 0.2 M mannitol) with 1% cellulase 0.1% pectolyase, and 0.1% dreisalase overnight, in the dark, at room temperature. Released protoplasts are filtered through a 150  $\mu\text{m}$  mesh and pelleted by centrifugation (100 x g, 5 minutes), then washed twice in plasmolyzing solution. Protoplasts are counted using a double chambered hemocytometer. DNA is transfected into the protoplasts by PEG treatment as described by Nunberg and Thomas (1993 Methods in Plant Molecular Biology and Biotechnology, B.R. Glick and J.E. Thompson, eds. pp 241-248) using  $10^6$  protoplasts and 50-70 ug of DNA from the above construct. Protoplasts are cultured in 5 ml of MS medium supplemented with 0.2 M mannitol and 3  $\mu\text{M}$  2, 4-D for 48 hours in the dark with shaking. Tobacco is transformed with the same  $\Delta 6$ -desaturase expression construct by following the method of Example 13.

In order to express the subject evening primrose cDNA in pBI121 (Jefferson et al. 1987 EMLBO J. 6:3901-3907), the following manipulations are performed:

The plasmid pBI121 is digested with EcoICR I (Promega) or Ecl 136 II (NEB) and Xba I which excises the GUS coding region and leaves the 35S promoter and NOS terminator intact. The evening primrose  $\Delta 6$ -desaturase cDNA is excised from pIB9748-4 by digestion with Xba I and Xho I. The Xho I end is made blunt by use of the Klenow fragment. The excised gene is then

cloned into the Xba I/Eco ICR I sites of pBI121. The  
resulting construct is used to transform *Arabidopsis*  
*thaliana* via *Agrobacterium* according to standard  
protocols (Bechtold N., Ellis. J., and Pelletier, G  
1993 C.R. Acad Sci Paris 316:1194-1199). Carrot and  
tobacco are transformed as described above.

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